Effect of fibronectin on the binding of antithrombin III to immobilized heparin

Youngro Byun,[1] Harvey A. Jacobs,[1] Jan Feijen,[2] and Sung Wan Kim[1,*]

[1]Department of Pharmaceutics and Pharmaceutical Chemistry, and Center for Controlled Chemical Delivery, The University of Utah, Salt Lake City, Utah; [2]Department of Chemical Technology, University of Twente, Enschede, The Netherlands

An objective of this research is to verify the mechanism of anticoagulant activity of surface-immobilized heparin in the presence of plasma proteins. The competition and binding interaction between immobilized heparin and antithrombin III (ATIII)/thrombin have been described *in vitro*. However, the strong ionic character of heparin leads to its specific and nonspecific binding with many other plasma proteins. Most notably, fibronectin contains six active binding sites for heparin which may interfere with the subsequent binding of heparin with ATIII or thrombin.

Heparin was covalently immobilized through polyethylene oxide (PEO) hydrophilic spacer groups onto a model surface synthesized by random copolymerization of styrene and p-aminostyrene. The binding interaction of immobilized heparin with ATIII was then determined in the presence of different fibronectin concentrations. The binding interaction was studied by first binding immobilized heparin with ATIII, followed by the introduction of fibronectin; heparin binding with fibronectin, followed by incubation with ATIII, and simultaneous incubation of surface immobilized heparin with ATIII and fibronectin. The extent of ATIII binding to heparin in each experiment was assayed using a chromogenic substrate for ATIII, S-2238.

The results of this study demonstrate that the displacement of ATIII from immobilized heparin was proportional to the fibronectin concentration, and was reversible. Furthermore, the binding sequence did not play a role in the final concentration of ATIII bound to immobilized heparin.

INTRODUCTION

Heparin is a polydispersed anionic polysaccharide molecule with a molecular weight ranging between 6000 and 35,000. Heparin binds and catalyzes the interaction of plasma proteins involved in the intrinsic and extrinsic clotting cascade, especially antithrombin III (ATIII). ATIII is the natural antagonist for thrombin, the protein which enzymatically cleaves fibrinogen to form the fibrin clot.

Rosenberg and Damus[1] described the overall mechanism by which thrombin is inactivated by ATIII. These investigators showed that ATIII neutralizes thrombin by forming a 1:1 stoichiometric complex via a reactive site (arginine)–active center (serine) interaction. The complex formation occurs at a relatively slow rate in the absence of heparin. However, heparin binds to lysine residues on ATIII, thereby accelerating the inhibition of thrombin.

*To whom correspondence should be addressed at Department of Pharmaceutics and Pharmaceutical Chemistry, and Center for Controlled Chemical Delivery, Biomedical Polymers Building (#570), Room 205, The University of Utah, Salt Lake City, UT 84112.

Due to the high molecular weight and ionic character of heparin, it has been studied for its binding interaction with other plasma proteins.[2,3] Specifically, heparin binding to plasma proteins has been divided into three classes, depending on the type and extent of binding interaction. The first class includes specific binding of ATIII to heparin, resulting in a conformational change in the protein and catalyzing its anticoagulant activity. The second class of binding interaction includes molecules such as heparin cofactor II,[4] lipoprotein,[5] histidine-rich glycoprotein,[6] and PF4.[7] The binding of this group is dependent on the charge density of heparin and protein configuration. The third class of binding includes proteins such as fibronectin,[8] thrombin, and vitronectin.[9] This binding interaction to heparin depends on the charge density and the chain length of heparin.

In previous research performed in our laboratory, Winterton et al.[10] studied the adsorption of fibronectin, albumin, and fibrinogen onto surface-immobilized heparin. They showed that both albumin and fibrinogen had no binding affinity to heparin at physiological pH. However, human plasma fibronectin was shown to bind to solution heparin and immobi-

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lized heparin. Furthermore, fibronectin was shown to have six active binding sites for heparin which may be sterically blocked in some adsorbed states.

The anticoagulant action of heparin immobilized onto polymer surfaces using grafted hydrophilic spacer groups has been reported[11,12] and the binding mechanisms of immobilized heparin with ATIII and thrombin were also delineated *in vitro*.[13,14] However, since heparin (both immobilized and solution) is able to bind other plasma proteins, especially *in vivo*, studies defining the competitive binding between ATIII and plasma proteins is necessary. As mentioned, fibronectin is a prominent plasma protein with specific binding sites for heparin, and plays a vital role in hemostasis. Therefore, the ability of immobilized heparin to bind to ATIII in the presence of fibronectin *in vitro* may help explain the anticoagulant mechanism of immobilized heparin *in vivo*.

EXPERIMENTAL PROCEDURES

Heparinized surfaces

Heparin was immobilized onto a surface via a hydrophilic spacer, as shown in Figure 1. Random copolymers of styrene (Aldrich Chemicals, Milwaukee, WI) and p-aminostyrene (Polysciences, Warrington, PA) were synthesized as the polymer substrate. The optimum composition ratio of styrene and p-aminostyrene was 74/26, as defined in previous research.[13,14] The polymer substrate was then coated onto glass beads (100 ± 10 μm diameter, Ferro-Cataphote Co., Jackson, MS) for subsequent polyethylene oxide (PEO) and heparin coupling.

PEO was used as a hydrophilic spacer group to couple heparin to the polymer surface. Hydroxyl groups of PEO (Sigma Chemical Co., St. Louis, MO) (MW 3400) were modified with tolylene diisocyanate (TDI, Kodak, Co., Rochester, NY) to generate isocyanate-reactive terminal groups. PEO spacer was grafted onto the polymer substrate through a chemical coupling reaction between the isocyanate group of modified PEO spacer and the $-NH_2$ group of p-aminostyrene. The surface density of grafted spacer was determined to be 16.3 pmol/cm^2, as reported in a previous study.[13,14]

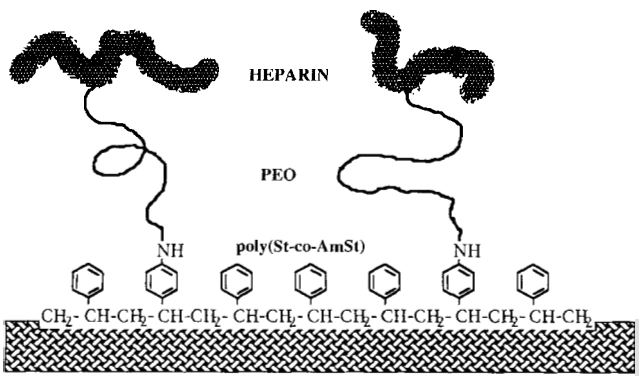

Figure 1. Heparin immobilization onto the polymer surface.

Low-molecular-weight heparin (Hepar Industries, Inc., Franklin, OH), with a molecular weight of 6000 and an anticlotting activity of 95 USP U/mg (Factor Xa), was used in these experiments. This heparin was fractionated on an ATIII-affinity column to obtain high-ATIII-affinity heparin. Approximately 12% of the initial heparin concentration was isolated in the high-affinity fraction, resulting in an absolute activity of 323 USP U/mg (FXa), nearly 3.4 times the activity of the unfractionated heparin.[13,14]

This high ATIII affinity heparin fraction was immobilized onto the PEO spacer by a coupling reaction between isocyanate groups of the PEO spacer and the amine or hydroxyl groups of heparin. The surface density of immobilized heparin was 13.7 pmol/cm^2, as cited in a previous report.[14] The binding mole ratio of immobilized heparin to PEO spacer was 0.91:1, implying that nearly one PEO group was coupled with one heparin molecule.[14]

Purification of fibronectin

Fibronectin was isolated from human plasma by affinity chromatography on a gelatin-Sepharose column at room temperature. Phenylmethysulfonylfluoride (0.1 m*M*) was added as a protease inhibitor to plasma, as well as to all buffers. Tris buffer (pH 7.4) included 10 m*M* EDTA, 0.02% NaN_3, 5 m*M* EACA (ε-amino capric acid), and 1 m*M* benzamidine. Plasma (250 mL, Bloodbank Twente-Achterhoek, Enschede, The Netherlands) was centrifuged at 300 *g* for 30 min. This plasma was eluted over a Sepharose 4B column (500 mL), and subsequently applied to a 125 mL column of gelatin-Sepharose 4B. The gelatin-Sepharose 4B column was washed with 50 m*M* Tris-HCl buffer for 3 h at a flow rate of 75 mL/h. After washing with 50 m*M* Tris-HCl buffer overnight, the column was washed with 1*M* NaCl in 50 m*M* Tris-HCl buffer (250 mL) to eliminate any nonspecifically bound fibronectin. The flow rate was 90 mL/h and the washing volume was 250 mL. After washing again with buffer for 3 hours (75 mL/h), the column was washed with 1*M* urea in 50 m*M* Tris-HCl buffer (250 mL) to elute the weak-affinity fibronectin. Finally, 4*M* urea in 50 m*M* Tris-HCl buffer was eluted with 20 mL/h flow rate. High-affinity fibronectin was eluted in the sixteenth through twenty-first volume (42 mL), based on UV absorbance at 280 nm. The fibronectin

solution was diluted to 0.5 mg/mL, dialyzed in PBS (pH 7.4) at 4°C, and stored at −30°C.

Glass treatment

Elgue et al.[15] reported that ATIII binding to heparin decreased by nearly 15% when the kinetic experiments were performed in plastic tubes, rather than siliconized glass tubes. Therefore, the glass vials used in subsequent kinetic experiments were treated with dichlorodimethylsiloxane (Kodak Co., Rochester, NY) to prevent nonspecific protein adsorption. Glass vials were soaked in 5% dichlorodimethylsiloxane in toluene at 25°C for 1 h, and then washed three times in pure ethanol, followed by washing in purified distilled water.

Chromogenic substrate assay for ATIII

The concentration of ATIII in all binding experiments was determined by a two-step chromogenic substrate assay using S-2238 (Pharmacia/Hepar, Franklin, OH). The heparin-immobilized beads were incubated in 2 mL of the various ATIII and fibronectin buffer solutions. An aliquot of the sample solution (600 μL) was incubated with 100 μL of buffer solution containing heparin (3 USP U/mg/mL) at 37°C for 3 min. One hundred microliters of thrombin solution (2 IU/mL) was added to the mixture and mixed for 1 min. Then, 300 μl of S-2238 solution (25 mg/38.5 mL) was added. The reaction was quenched by adding 100 μL concentrated acetic acid. The reaction between S-2238 and free thrombin was monitored by measuring the enzymatic cleavage of S-2238 to release p-nitroanilide fragments (pNA, λ_{max} = 405 nm).

Binding of ATIII to immobilized heparin

The interaction of ATIII with this heparinized surface has been reported.[14] In these studies, 250 mg of polymer-PEO-heparin beads (~60 cm^2) were incubated with various concentrations (0–1 μ*M*) of ATIII solution. After 10 minutes (the time to obtain maximum binding interaction), the concentration of ATIII was assayed and a Scatchard plot was constructed. In this study, the binding constant between immobilized heparin and ATIII was determined to be (Ka) $9.58 \times 10^7\ M^{-1}$. Furthermore, nonspecific binding between ATIII and the control surface or heparin immobilized surface was negligible.[14]

Binding of fibronectin to heparin in ATIII solution

Two hundred fifty (250) mg (~60 cm^2) of heparin-immobilized beads and an ATIII concentration of 0.5 μg/mL were used in each study, while the concentration of fibronectin was 1 μg/mL and 100 μg/mL. The binding kinetics of fibronectin to heparin in the presence of ATIII were studied under three different conditions, as shown in Figure 2.

In the first study, the heparinized beads were incubated in a 1-mL solution of ATIII (0.5 μg/mL) for 10

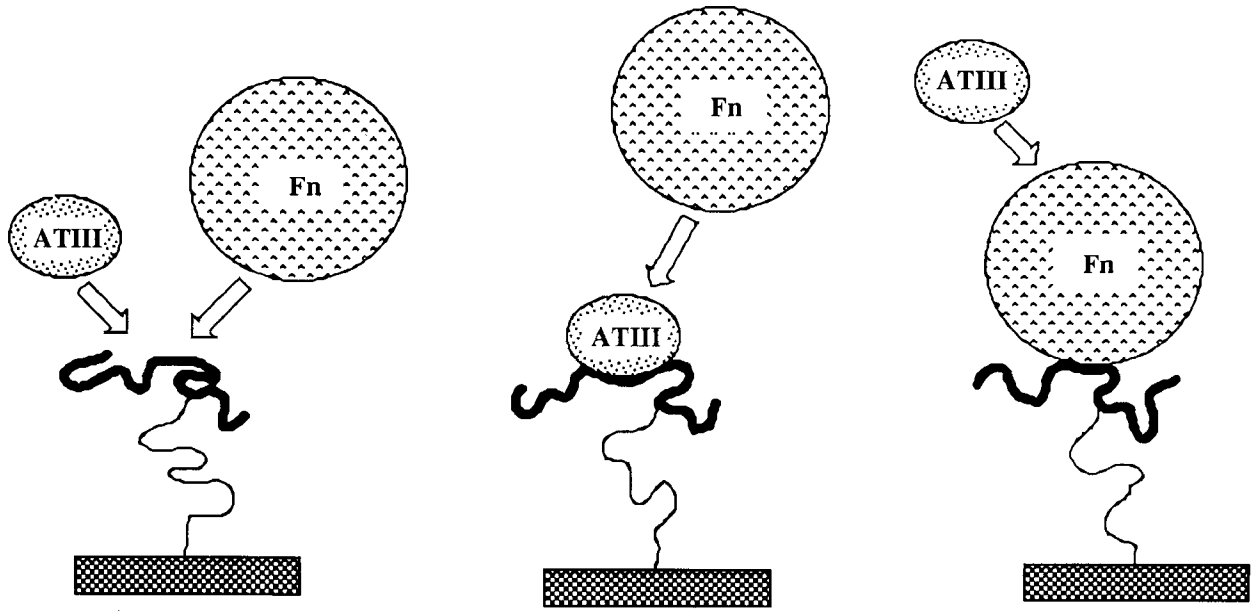

Figure 2. Summary of experiments representing the sequence of ATIII and fibronectin binding to the heparinized surface.

min. In separate experiments, 1 mL of the 1-μg/mL and 100-μg/mL fibronectin solutions were added, and after 5 minutes, the solutions were sampled for the equilibrium ATIII solution concentration using the S-2238 substrate assay. Increases in ATIII from initial conditions implied a desorption from the immobilized heparin surface and an exchange process with fibronectin.

In the second study, the heparin-immobilized beads were incubated in a mixed solution of ATIII (0.5 μg/mL) and fibronectin (1 μg/mL and 100 μg/mL) for 5 min. The bulk concentration of ATIII was then assayed.

Finally, fibronectin (1 μg/mL and 100 μg/mL) was incubated with heparin-immobilized beads for 5 min, followed by the addition of ATIII. In all cases, the change of ATIII in the bulk concentration was determined.

RESULTS AND DISCUSSION

The ability of surface-immobilized heparin to prevent material-induced thrombus formation both *in vitro* and *in vivo* is well known. The main approach undertaken by our laboratory focused on heparin immobilization using PEO spacer groups onto a variety of materials. The biological activity of spacer-immobilized heparin has been verified, and a PEO chain length of 3400 daltons demonstrated optimum anticoagulant activity of heparin.[11]

The current study dealing with fibronectin and ATIII interactions was part of a series investigating the interaction of spacer (PEO)-immobilized heparin with other proteins, including ATIII and thrombin. The polymer substrate (styrene/p-aminostyrene) was used as a model surface not for its biocompatible properties, but for its ease of synthesis and reproducibility. The amount of amine groups (p-aminostyrene content, 75:25 [Sty:p-amino Sty]) was selected based on experiments which demonstrated that optimum (~1:1) grafting of PEO groups occurred on the surface under these specific conditions. Thus, the amount of heparin groups immobilized onto the surface may not have been maximal in terms of other surfaces and other experiments, but it was optimal in the overall control and characterization of heparin coupling to PEO spacer groups (~.91:hep:PEO). Again, this particular model surface allowed comparable amounts of direct immobilized heparin and PEO spacer-immobilized heparin for protein binding comparisons.[13,14]

This previous research[13,14] described the binding interaction of spacer-immobilized heparin with ATIII and thrombin. This research determined that PEO spacer–immobilized heparin binds and interacts primarily with ATIII, and has low affinity to thrombin. This study reported that the binding constant of ATIII to immobilized heparin was $9.58 \times 10^6\ M^{-1}$,[14] which is similar to the binding constant of solution heparin to ATIII ($10 \times 10^6\ M^{-1}$).[16] Having this basic information led to initiate binding studies of other plasma proteins with immobilized heparin, in particular, fibronectin.

Fibronectin is a high-molecular-weight glycoprotein found in blood, other body fluids, cells, connective tissue, and platelets, and associated with basement membranes. Fibronectins are involved in cell–cell adhesion, wound healing, opsonic activity, platelet adherence, complement activation, and activation of the coagulation system. As mentioned, fibronectin has six ionic binding sites specific for heparin, and thus may interfere with ATIII binding to immobilized heparin. The binding interactions and competition between ATIII and fibronectin to immobilized heparin may therefore help to explain the biological effect exhibited by this molecule.

The ATIII concentration used in these studies of 0.5 μg/mL (~9 n*M*) is far below saturation conditions. During the ATIII binding kinetic studies,[14] ATIII concentrations between 0 and 1 μ*M* were used to construct the Scatchard plots. In plasma, the concentrations of ATIII and fibronectin are 100 ~ 200 μg/mL and 150 ~ 300 μg/mL, respectively. The concentration of ATIII in these studies was fixed at 0.5 μg/mL, while the concentration of fibronectin was 1 and 100 μg/mL. The ATIII:fibronectin ratio of 0.5 μg/mL to 1 μg/mL represents a mid-range of the physiological ratio of the proteins, while the 100-μg/mL fibronectin is far in excess.

The binding reactions of ATIII and fibronectin to immobilized heparin were studied under three different conditions. In the first experiment (a), heparinized beads were mixed and equilibrated with ATIII, and then fibronectin was added to the solution. In the second experiment (b), both ATIII and fibronectin were added to the heparinized beads simultaneously. Finally, in the third experiment (c), ATIII was added to the solution after the heparinized beads were mixed and equilibrated with fibronectin.

Figure 3 shows the change in the concentration of ATIII bound to immobilized heparin over time under experimental condition (a). In this study, the heparin-ATIII complex was formed by incubating heparin immobilized beads in 1 mL of a 0.5-μg/mL ATIII solution. One microgram (1 μg) fibronectin was added to this solution and the change in bulk ATIII concentration was measured. Using S-2238, the solution concentration of ATIII did not change. In this experiment, the concentration ratio of ATIII and fibronectin was similar to that found *in vivo* (in plasma). However, when 100 μg of fibronectin was added to the ATIII-bound heparin complex, ~64.2% of initially

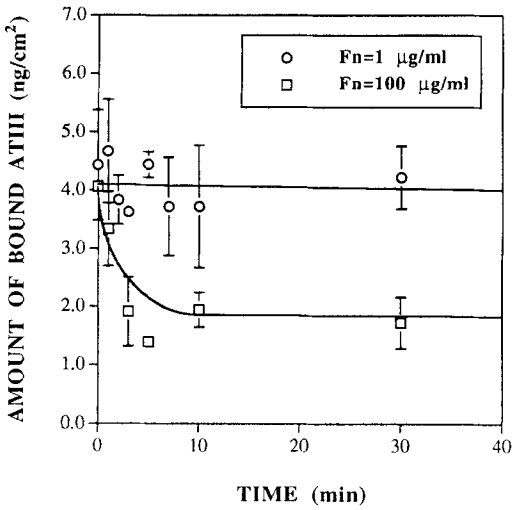

Figure 3. Binding interaction of fibronectin with ATIII bound to immobilized heparin as a function of time.

bound ATIII was displaced by fibronectin from the binding site of immobilized heparin. This exchange process between two proteins on immobilized heparin was equilibrated within 5 min. In this experiment, the ratio of fibronectin to ATIII concentration was ~100 times that found in plasma.

Figure 4 shows the desorption of bound ATIII for experimental condition (a) as a function of fibronectin concentration. At low fibronectin concentrations, ATIII was not displaced from heparin by fibronectin. However, as the fibronectin concentration increased, the desorption of bound ATIII increased, demonstrating exchange between ATIII and fibronectin for binding to immobilized heparin.

Figure 5 shows the concentrations of ATIII bound to immobilized heparin at equilibrium when studied under all the experimental conditions mentioned

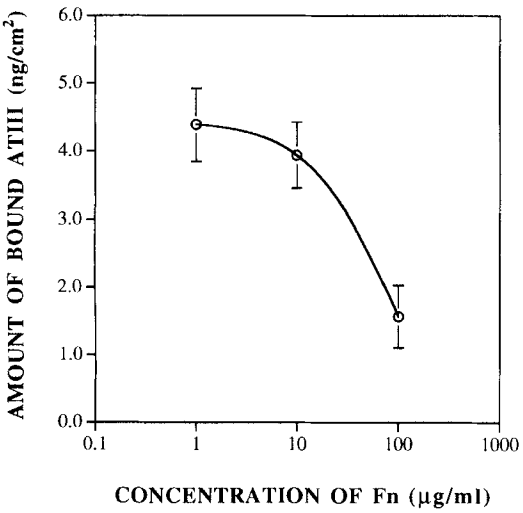

Figure 4. Binding interaction of fibronectin with ATIII bound to immobilized heparin as a function of fibronectin concentrations.

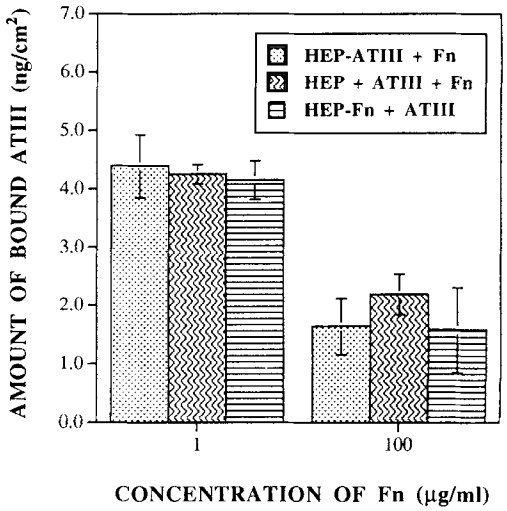

Figure 5. Overall evaluation of fibronectin competing with ATIII for immobilized heparin as a function of incubation sequence and fibronectin concentration.

above. Again, the concentration of ATIII was 0.5 μg/mL, and the initial concentrations of fibronectin were chosen as 1 and 100 μg/mL. The concentrations of ATIII bound to immobilized heparin under different conditions were nearly the same at equilibrium for all experimental conditions for each particular fibronectin concentration. Thus, the binding competition between ATIII and fibronectin to immobilized heparin depended on the bulk protein concentration.

CONCLUSION

In this study, the binding interactions between antithrombin III and fibronectin to spacer-immobilized heparin was studied. Extensive research has detailed the binding between ATIII and immobilized heparin *in vitro*. However, *in vivo* contact of immobilized drugs may involve binding competition between several proteins. Therefore, the effect of protein binding of immobilized heparin with ATIII in the presence of another primary plasma protein, fibronectin, was investigated. At protein concentrations representing physiological ratios, fibronectin did not displace bound ATIII from immobilized heparin. However, under excessive fibronectin concentration, ATIII was desorbed from heparin. This demonstrates that fibronectin can interfere with ATIII binding to heparin as a function of fibronectin concentration. This study may help extrapolate *in vitro* protein binding data of immobilized heparin to that of *in vivo* results.

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